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Source–sink relationship and photosynthesis in the horn-shaped gall and its host plant *Copaifera langsdorffii* Desf. (Fabaceae)

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Abstract

The horn-shaped gall of *Copaifera langsdorffii* is induced by an unidentified species of Diptera: Cecidomyiidae and stands out among the other gall morphotypes by its bizarre shape and high infestation level over the years. Its role as a sink of photoassimilates in comparison to the non-galled leaflets was assessed through the quantification of nitrogen, carbohydrates, pigment content and the apparent relative electron transport rate (ETR) during different developmental stages. The levels of nitrogen and starch decrease in galls as leaflets mature. Total soluble sugars and the water-soluble polysaccharide concentration were higher in galls, whereas, chlorophyll content and ETR values were higher in leaflets rather than in galls. However, the latter presented significantly higher concentrations of total carotenoids. The low nitrogen and chlorophyll contents are related to the low photosynthetic activity, indicating that the CO_2 assimilation in galls is insufficient to fully supply their metabolism, reinforcing it as a sink of photoassimilates. The amount of sugars allocated to the gall tissues corroborates this hypothesis, and is probably used as energy supply for both gall structure maintenance and herbivore nutrition. The reduced intercellular spaces on the horn-shaped gall structure imply little gas exchange and, thus, hypoxic conditions on the gall tissues. However, the incipient photosynthesis might be important to provide oxygen to the structure and avoid hypoxia, enabling gall metabolism.

Keywords: Carbohydrates; Carotenoids; Cecidomyiidae; Chlorophyll; ETR; Nitrogen

1. Introduction

Galls are symmetric structures induced by insects, which develop through cell redifferentiation on the host plant tissues (Oliveira and Isaias, 2010a). The gall formation protects the gall-inducing insect against abiotic factors such as high temperatures, drought and rain, and biotic factors like predators and diseases, besides providing an adequate microenvironment for its development (Price et al., 1987). In addition, the host plant ensures nutritional resources for the new formed organ, the gall (Bronner, 1992; Mani, 1964; Price et al., 1987). In general, galls occur in reactive tissues of the host plants (Weiss et al., 1988), and modify their morphogenetical pattern (Isaias et al., 2011; Oliveira and Isaias, 2010a). More than morphological alterations, gall development changes the source–sink relationships between it and the adjacent tissues. The manipulation of the plant tissues by the gall inducer alters the cellular carbohydrate metabolism, inducing gall tissues to act as a sink to attend its energetic demands (Wingler and Roitsch, 2008). This effect leads to an accumulation of soluble compounds like sugars and nitrogen, which confers advantages to the galling herbivores for they improve the nutritional quality of their microenvironment (Hartley, 1998). In Cecidomyiidae galls, a gradient of starch can be verified in the reserve tissue towards the larval chamber, culminating in the accumulation of simple sugars

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in the nutritive cells closest to the gall inducer (Bronner, 1992). Besides carbon (Bronner, 1992), the galls can accumulate high levels of proteins, which are related to the high stress caused by the oviposition and feeding activity of the galling herbivore (Oliveira et al., 2010; Oliveira and Isaias, 2010b). Young tissues tend to be more reactive to gall formation stimuli than mature ones (Rohfritsch, 1992), probably because they function as sinks of photoassimilates, with higher nutrient availability and a greater potential for division and differentiation (Weiss et al., 1988).

Another aspect to be considered is that gall induction and development expose host plant tissues to intense oxidative stress (Hartley, 1998; Isaias et al., 2011; Oliveira and Isaias, 2010b; Oliveira et al., 2011a), which can cause either negative (Andersen and Mizell, 1987; Florentine et al., 2005; Larson, 1998) or positive effects on the photosynthesis of their host organs (Fay et al., 1996; Oliveira et al., 2011b). These effects may be neutral, as in the galls induced by aphids and psyllids (Larson, 1998; Oliveira et al., 2011b). Some studies show that cell differentiation in galls may end up in structural modifications in the chlorophyllian tissues, alterations in the photosynthetic pigment contents, and damage to Photosystem II (PSII) as well as changes in gas exchange rates (Florentine et al., 2005; Oliveira et al., 2011b; Yang et al., 2003).

Taking the nutritional hypothesis proposed by Price et al. (1987) for granted, the horn-shaped gall in its mature phase should accumulate high levels of carbohydrates and nitrogen. It should also have low chlorophyll content and present low activity of the photosynthetic apparatus. To test these assumptions, this study aimed to: (1) characterize the gall chlorophyllian tissue at mature stage, (2) quantify the chlorophyll content in mature galls and non-galled leaflets, (3) analyze the levels of carbohydrates and nitrogen in leaflets and in galls at different developmental stages, and (4) investigate the photosynthesis in galls and non-galled tissues by measurements of chlorophyll fluorescence.

The horn-shaped gall of the superhost *Copaifera langsdorffii* Desf. (Fabaceae) was chosen as a model of study. It is induced in young leaflets by an unidentified species of Diptera: Cecidomyiidae (Oliveira et al., 2011a), and is distinguishable by its peculiar shape and abundance (Oliveira et al., 2012).

2. Material and methods

Samples of non-galled leaflets and horn-shaped galls both in young and mature phases ($n \geq 20$) were collected in a population of *C. langsdorffii* Desf. located in an ironstone outcrop area so-called "canga" (20° 05′ 35″S, 43° 59′ 01″W), at Serra da Calçada, Minas Gerais, Brazil. "Canga" is a type of Savannah with predominantly herbaceous vegetation growing on ferruginous-rocky, very shallow and nutrient-poor soil (Jacobi et al., 2007). The gall cycle was accompanied between February 2008 and January 2009.

2.1. Anatomical characterization

Galls fixed in Karnovsky in phosphate buffer 0.1 M (pH 7.2) (Karnovsky, 1965, modified) were dehydrated in *n*-butyl series and embedded in Paraplast® (Kraus and Arduin, 1997). The material was cross-sectioned in a rotative microtome (12 μm) (Jung Biocut 2035), hydrated in ethanol series, stained with toluidine blue (O'Brien et al., 1965) and mounted in Kaiser's jelly glycerin (Kraus and Arduin, 1997).

2.2. Contents of carbohydrates and nitrogen

The samples of young and mature non-galled leaflets and galls were refrigerated in the field. In laboratory, their metabolic activity was interrupted by 30 second-exposure to high potential microwaves (Marur and Sodek, 1995). After this, they were oven-dried at ~60 °C, macerated in a mortar and stored free of moisture. The samples were extracted with methanol:chloroform:water (MCW) (12:5:3 v/v/v) based on Bielski and Turner (1966), the aqueous phase was used for the analyses of total soluble sugar (TSS). The residue obtained after MCW extraction was extracted twice with 10% ethanol to solubilize water-soluble polysaccharides (WSP) (Shannon, 1968), and then with 30% $HClO_4$ to extract starch (McCready et al., 1950). The dosage of carbohydrates was performed by phenolic–sulfuric method (Dubois et al., 1956) modified by Chow and Landhäusser (2004), using glucose as standard. The quantification of nitrogen contents were obtained by the Kjeldahl method (Tedesco et al., 1995). The samples were digested in concentrated sulfuric acid and the residue distilled (distiller Tecnal TE-0363). The ammonia released in the form of NH_4OH was trapped in 2% boric acid and titrated against 0.02 N hydrochloric acid previously standardized.

2.3. Photosynthetic pigments and chlorophyll a fluorescence

For characterization of the chlorophyllian tissue, histological slides with fresh leaflet and gall samples in the maturation phase (gall height = 9.5 ± 2.1 mm) (n = 30) were prepared. The sections were handmade and observed in light microscope. The photosynthetic pigments of non-galled leaflets and mature horn-shaped galls were extracted in 80% acetone (v/v), using weighted disks of 1 cm^2. The extracts were read in a spectrophotometer and chlorophyll and carotenoid quantification followed the equations proposed by Lichtenthaler and Wellburn (1983), with the values expressed in μg g^{-1} of fresh mass (FM).

A photosynthesis yield analyzer (Mini-PAM, Waltz, Germany) was used to measure chlorophyll *a* fluorescence. The photosynthetic performance in function of increasing levels of light was given between 9:00 and 11:00 A.M. in galls and non-galled leaflets of five individuals of *C. langsdorffii*, using the program of the equipment with increasing levels of light for 4 min in eight stages of 30 s each. At the end of each level of light, a saturating pulse was applied for determination of fluorescence parameters. The apparent relative electron transport rate (ETR) was determined by $0.5\ (\Delta F/F'm).\ DPF\ 0.84$ (Lüttge et al., 1998), where 0.5 was a factor considering the luminous excitation of the two photosystems, *DPF* corresponded to the density of photosynthetically active photon flux and 0.84 means that only 84% of irradiance was absorbed by the leaf.

2.4. Data analysis

The normal distribution was verified for the results of nitrogen and carbohydrate contents. ANOVA was used for parametric data and Wilcoxon for non-parametric ones with the softwares JMP® 5.0 Software (Sas Institute, Inc. 2002, The statistical discovery software) and Graphpad Prism® 5.0 (GraphPad Software, Inc. 1992–2009). Means were compared by Tukey and t-Student tests ($p<0.05$).

3. Results

3.1. Structural analysis

The young leaflets are reddish, becoming dark green at maturity, while the horn-shaped galls are pilous and predominantly reddish when young, turning brownish and glabrous when mature (Fig. 1a). This covering gall is pedunculated and closed; may occur either isolated or grouped, mainly on the abaxial leaflet surface. Each gall has one larval chamber that shelters a single insect (Fig. 1b). Anatomical analyses evidenced tissue zonation around the larval chamber, with the innermost layers corresponding to the nutritive tissue and the outer layers to the reserve parenchyma (Fig. 1b). Gall is mainly covered by periderm. The cells around the larval chamber and in the gall cortex are compact, with reduced intercellular spaces (Fig. 1c). The non-galled leaflets present abundant chlorophyllian tissue arranged in palisade and spongy parenchymas (Fig. 2a). In the horn-shaped gall, chloroplasts occur only in the cells from the outer cortex (Fig. 2b), but are still scarce when compared to the cells from the non-galled leaflet (Fig. 2c and d).

3.2. Carbohydrate and nitrogen contents

The starch content was significantly higher in young non-galled leaflets (94.53±26.25 mg g^{-1} DM), when compared to mature leaflets (44.99±6.57 mg g^{-1} DM), young and mature galls (29.0±9.6 and 44.6±11.7 mg g^{-1} DM, respectively) (Fig. 3). On the other hand, the TSS (182.6±28.4 mg g^{-1} DM in young galls and 170.9±64.4 mg g^{-1} DM in mature galls) and the WSP contents (32.6±11.3 mg g^{-1} DM in young galls and 28.1±7.2 mg g^{-1} DM in mature galls) were higher in galls than in the non-galled leaflets (60.45 mg g^{-1} DM of TSS in young and 45.9 mg g^{-1} DM in mature leaflets; 4.8 mg g^{-1} DM of WSP in young and 5.0 mg g^{-1} DM in mature leaflets) (Fig. 3). The nitrogen content was higher in young non-galled leaflets (~3% of DM) than in mature ones (~2.5% of DM) (Fig. 4), and even higher than that of galls, which did not exceed 1%. The levels of nitrogen did not differ significantly between young and mature galls (0.58% and 0.75% of DM, respectively).

3.3. Photosynthetic pigments and fluorescence of chlorophyll a

The total chlorophyll content (mg g^{-1} FW) was 23-fold lower in galls when compared to non-galled leaflets, while the concentration of carotenoids was 12-fold lower. The chlorophyll *a/b* ratio was lower in galls than in non-galled leaflets, while the carotenoids/total chlorophyll ratio was higher for gall tissues (Table 1). The PSII maximum electron transport rate for galls was approximately 7-fold lower than that of non-galled leaflets and the PPFD to get 50% of maximum ETR and saturation was 3-fold lower for gall tissues (Table 1).

4. Discussion

During the development of the horn-shaped gall, the redifferentiation of cells leads to the formation of reserve and nutritive tissues, as described by Oliveira et al. (2011a). The lack of intercellular spaces in the gall tissue, a typical consequence of the hypertrophy and homogenization of the parenchymatic cells during gall development, could be important to maintain the hydric content, by avoiding excessive loss of water (Kraus, 2009). The suberized dermal system, the absence of stomata, and the lack of intercellular spaces can limit oxygen diffusion (Heldt

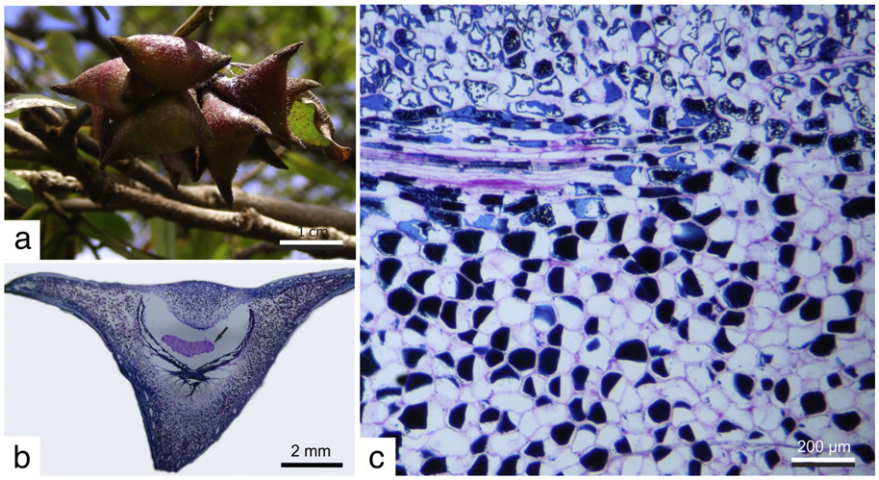

Fig. 1. (a) Morphological aspects of horn-shaped gall of *Copaifera langsdorffii*. (b) Gall in transverse section evidencing the gall inducer in the larval chamber (arrow). (c) Detail of gall tissues close to the larval chamber on mature gall evidencing the lack of intercellular spaces.

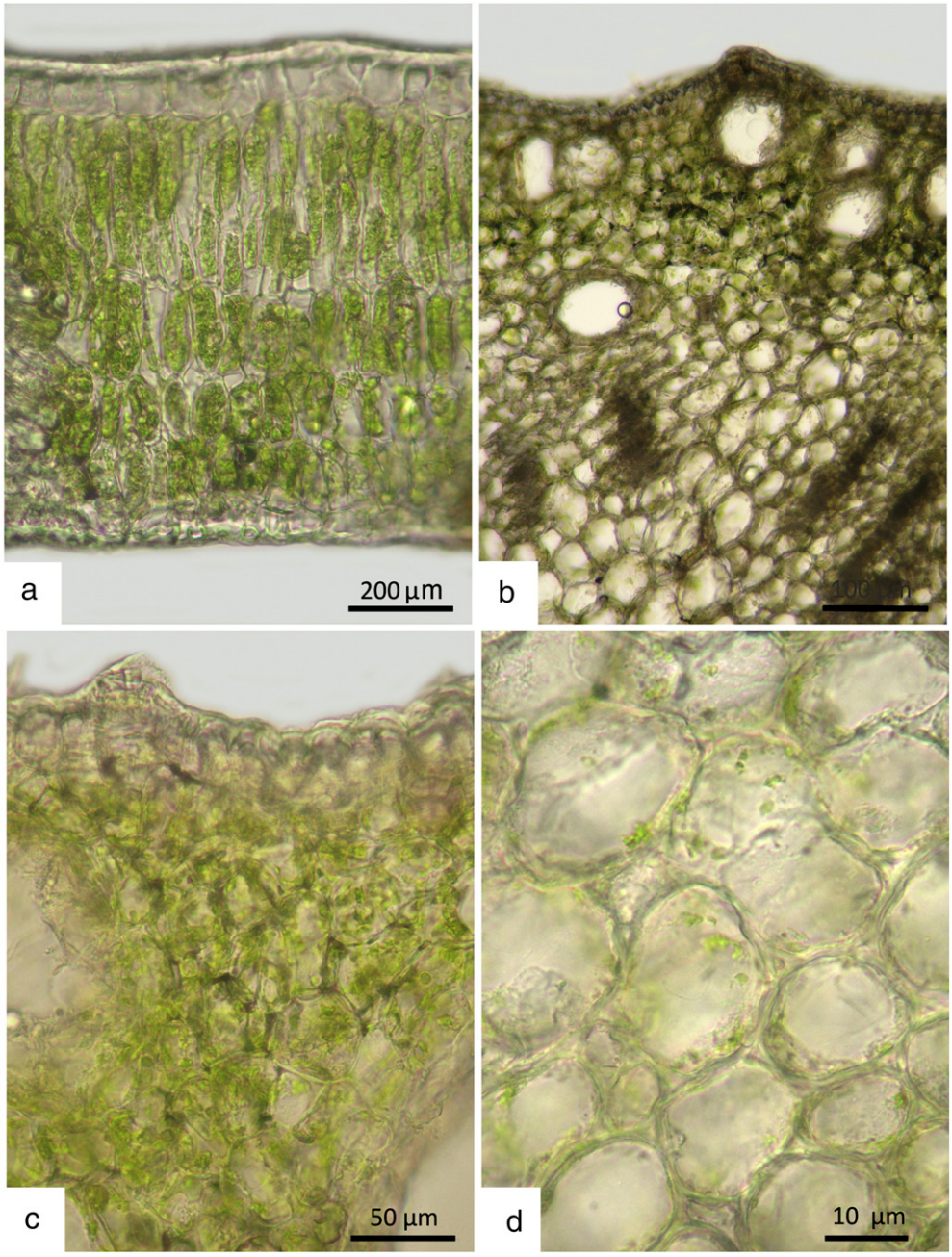

Fig. 2. Non-galled leaflet and horn-shaped gall of *Copaifera langsdorffii* in transverse sections. (a) Non-galled leaflet with abundant chloroplasts in the mesophyll. (b) Horn-shaped gall, with chloroplasts restrict to the outer parenchyma. (c) Parenchyma of the horn-shaped gall and (d) detail of the cells evidencing chloroplast position.

and Piechulla, 2010). Therefore, despite the low production of oxygen inferred by the ETR values, the levels of oxygen produced in gall tissues could contribute to the maintenance of aerobic metabolism in the structure. The role of photosynthesis favoring the aerobic environment was already reported in aerial roots of Orchidaceae (Moreira et al., 2009), in legume fruits (Lemos-Filho and Isaias, 2004), and in other non-foliar tissues (Aschan and Pfanz, 2003).

The nitrogen content in the horn-shaped galls was lower than in non-galled leaflets, similarly to the data of Hartley (1998) in most of the 33 distinct studied galls. This result does not corroborate the nutritional hypothesis (Price et al., 1987), which assumes higher concentrations of protein, phosphate and lipids in gall sites by the translocation of resources from the host organ or the whole plant. Levels of nitrogen and secondary compounds in gall tissues are usually different from those found in tissues near the site of their development (Hartley, 1998). High levels of soluble components such as sugars and amino acids are found in the nutritive tissues, and the levels of soluble nitrogen may be higher in galled than in non-galled tissues of the host plant. However, the total nitrogen content may be low due to spatial variation, for they have been allocated to other tissues, and may be time-dependent due to the phenological activity in which the plant or plant organ are in (Hartley, 1998). A high level of

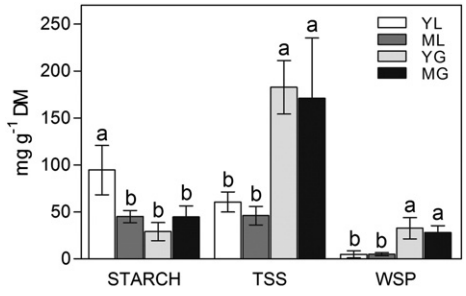

Fig. 3. Carbohydrate contents (mg g^{-1}) of non-galled leaflets and horn-shaped galls of *Copaifera langsdorffii*. Contents of starch, total soluble sugars (TSS) and water-soluble polysaccharides (WSP) in young leaflets (YL), mature leaflets (ML), young galls (YG) and mature galls (MG). Values indicated by the same letters do not differ significantly by t-Student test ($p<0.05$).

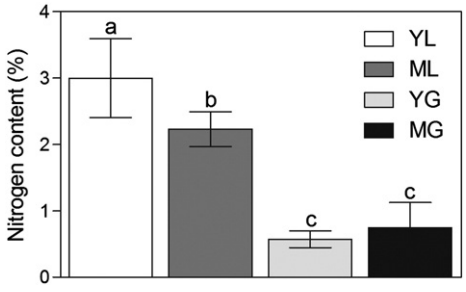

Fig. 4. Contents of nitrogen (% of dry mass) of non-galled leaflets and horn-shaped galls of *Copaifera langsdorffii* in young leaflets (YL), mature leaflets (ML), young galls (YG) and mature galls (MG). Values indicated by the same letters do not differ significantly by the Tukey test ($p<0.05$).

nitrogen in young leaves, as measured for *C. langsdorffii*, denotes their intense protein metabolism (Heldt and Piechulla, 2010). Thus, the galls should have peculiar ways of improving nutrition of the galling insect, such as the accumulation of nitrogen compounds histochemically evidenced in the tissue layers next to the larval chamber of the horn-shaped gall (Oliveira et al., 2011a). Even with the high metabolic activity, rapid growth and weight gain, the levels of nitrogen content were similar either in young and mature gall tissues. In the horn-shaped gall, the low nitrogen content may be a consequence of the low amount of chlorophyll, and possibly to the low content of rubisco. These features are corroborated by the lower values of ETR of galls when compared to non-galled leaflets.

The young leaflets of *C. langsdorffii* presented high levels of starch when compared to mature leaflets and galls. The lower level of starch in mature leaves can be caused either by its depolymerization and absorption for the high metabolic activity or by the formation of sinks, with relocation to other plant parts (Heldt and Piechulla, 2010). Despite the similarity of starch levels between the horn-shaped galls and mature leaflets, the levels of total soluble sugars in galls were 3-fold higher. The levels of TSS in the tissues of the horn-shaped gall together with the lower photosynthetic activity, herein indicated by the lower values of ETR, corroborate the hypothesis of the galls as sinks of assimilates. The data on sugar accumulation complements the findings of Oliveira et al. (2011a), who histochemically detected starch grains in the reserve tissue, and reducing sugars in the nutritive tissue. The highest concentration of total soluble sugars (TSS) in the horn-shaped galls should indicate that the intake of carbohydrates is necessary as energy source for the gall development. Also, the metabolism of the galling herbivore should be high, generating a sink of photoassimilates to gall site (Hartley, 1998; Oliveira and Isaias, 2009). Although, this does not seem to be a pattern, since for *Sellaginella*, a higher concentration of TSS was found in non-galled branches when compared to galls (Patra et al., 2010). Accompanying the total content of TSS, there was a high content of water-soluble polysaccharides (WSP), represented by pectins and structural carbohydrates. This concentration may be related to an increase of pectic matrix in the cells of the horn-shaped gall, showing the importance of the dynamics of cell walls for shape, growth, adhesion, mechanical properties, signaling and for the formation of intercellular spaces, as pointed out by Knox (1992). The activity of the gall inducing insect may cause changes on photosynthesis, with damage to the PSII, changes in gas exchange, and reduction in pigments (Florentine et al., 2005; Yang et al., 2003). In this study, there was a reduction of the photosynthetic activity only on the horn-shaped gall. Other studies have shown positive effects under the photosynthetic apparatus, with better rates of net photosynthesis (Fay et al., 1993) or maintenance of the electron transport rates (Oliveira et al., 2011b). Moreover, these positive effects were found for intralaminar galls, which form a structural *continuum* with the chlorophyllian parenchyma of their host leaves. On the horn-shaped gall, there was a considerable increase in the total volume of the structure compared to the non-galled leaflets, with few scattered chloroplasts in the reserve tissue. Even though there is a certain concentration of plastids restricted to the periphery of the gall, the photosynthetic activity is low, with a saturation of photosystem II under low photosynthetically active radiation (PAR). It is already known that gall development may cause reduction in photosynthetic pigment contents (Oliveira et al., 2011b; Yang et al., 2003). Thus, photosynthetic activity in the

Table 1
Pigment contents (chlorophylls *a* and *b* and carotenoids) in leaflets and horn-shaped galls of *Copaifera langsdorffii*. Values followed by the same letters do not differ significantly at $p<0.05$ level by the Wilcoxon ($n=30\pm$SE for pigment contents, $n=5\pm$SE for ETR dates).

	Total chl	Carotenoids	Chl *a/b*	Carot/Chl	ETR_{MAX}	PAR 1/2 ETR_{MAX}	PAR 0.9 ETR_{MAX}
	mg g^{-1} FW	mg g^{-1} FW			μmol m^{-2} s^{-1}	μmol m^{-2} s^{-1}	μmol m^{-2} s^{-1}
Leaflets	1.60 ± 0.072^a	0.38 ± 0.014^a	3.07 ± 0.036^a	0.24 ± 0.004^b	85.6 ± 8.8^a	238.9 ± 39.9^a	792.6 ± 132.8^a
Galls	0.06 ± 0.002^b	0.03 ± 0.001^b	2.43 ± 0.082^b	0.48 ± 0.017^a	12.4 ± 2.1^b	77.5 ± 28.9^b	257.1 ± 95.9^b
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.009	<0.163	<0.163

horn-shaped gall is insufficient to guarantee the supply of carbohydrates needed to maintain the functioning of cell machinery, reinforcing its character as a sink even at mature stage. Nevertheless, this gall photosynthesis may play an important role in sustaining the aerobic metabolism, since they have few intercellular spaces.

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